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Review

Alcohol, microbiome, life style influence alcohol and non-alcoholic organ damage

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ABSTRACT

This paper is based upon the "8th Charles Lieber's Satellite Symposium" organized by Manuela G. Neuman at the Research Society on Alcoholism Annual Meeting, on June 25, 2016 at New Orleans, Louisiana, USA.

The integrative symposium investigated different aspects of alcohol-induced liver disease (ALD) as well as non-alcohol-induced liver disease (NAFLD) and possible repair. We revealed the basic aspects of alcohol metabolism that may be responsible for the development of liver disease as well as the factors that determine the amount, frequency and which type of alcohol misuse leads to liver and gastrointestinal diseases. We aimed to (1) describe the immuno-pathology of ALD, (2) examine the role of genetics in the development of alcoholic hepatitis (ASH) and NAFLD, (3) propose diagnostic markers of ASH and non-alcoholic steatohepatitis (NASH), (4) examine age and ethnic differences as well as analyze the validity of some models, (5) develop common research tools and biomarkers to study alcohol-induced effects, 6) examine the role of alcohol in oral health and colon and gastro-intestinal cancer and (7) focus on factors that aggravate the severity of organ-damage.

The present review includes pre-clinical, translational and clinical research that characterizes ALD and NAFLD. Strong clinical and experimental evidence lead to recognition of the key toxic role of alcohol in the pathogenesis of ALD with simple fatty infiltrations and chronic alcoholic hepatitis with hepatic fibrosis or cirrhosis. These latter

Abbreviations: ADH, alcohol dehydrogenase; AH, acute alcoholic hepatitis; ALD, alcoholic liver disease; ALT, alanine aminotransferase; ARIC, Atherosclerosis Risk in Communities; ASH, alcoholic steato-hepatitis; AST, aspartate aminotransferase; ATM, ataxia-telangiectasis-mutated; ATR, ataxia and rad3 related; AUDIT, Alcohol Use Disorders Identification; CDT, carbohydrate-deficient transferrin; Chk, checkpoint kinase; CYP, cytochrome p450; FFA, free fatty acid; FXR, farsenoid X receptor; GAA, guanidino-acetate; GAMT, guanidino-acetate methyltransferase; γGT, gamma glutamyl transpeptidase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HDL, high density lipoprotein; HFD, high fat diet; 4-HNE, 4-hydroxynonenal; IARC, International Agency for Research on Cancer; IGF, insulin-like growth factor; IL, interleukin; LA, linoleic acid; LDL, low density lipoprotein; LGG, *Lactobacillus rhamnosus* GG; LPS, lipopolysaccharide; MCV, mean corpuscular volume of erythrocytes; MDB, Mallory-Denk body; MDF, Maddrey discriminator function; MELD, Model for End-Stage Liver Disease; MEOS, microsomal ethanol oxidizing system; MR, Mendelian Randomization; MRI, magnetic resonance imaging; NAFLD, non-alcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PAMPS, pathogen-associated molecular patterns; ROS, reactive oxygen species; SAH, S-adenosyl-homocysteine; SAM, S-adenosyl-methionine; SIBO, small intestinal bacterial overgrowth; SNAP, single nucleotide polymorphisms; SF, saturated fat; TIMP, tissue inhibitor of metalloproteinase; TLR, toll like receptor; TNF, tumor necrosis factor; USF, unsaturated fat.

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Mithocondrion
Oral health

stages may also be associated with a number of cellular and histological changes, including the presence of Mallory's hyaline, megamitochondria, or perivenular and perisinusoidal fibrosis. Genetic polymorphisms of ethanol metabolizing enzymes and cytochrome p450 (CYP) 2E1 activation may change the severity of ASH and NASH. Other risk factors such as its co-morbidities with chronic viral hepatitis in the presence or absence of human deficiency virus were discussed. Dysregulation of metabolism, as a result of ethanol exposure, in the intestine leads to colon carcinogenesis. The hepatotoxic effects of ethanol undermine the contribution of malnutrition to the liver injury. Dietary interventions such as micro and macronutrients, as well as changes to the microbiota have been suggested. The clinical aspects of NASH, as part of the metabolic syndrome in the aging population, have been presented. The symposium addressed mechanisms and biomarkers of alcohol induced damage to different organs, as well as the role of the microbiome in this dialog. The microbiota regulates and acts as a key element in harmonizing immune responses at intestinal mucosal surfaces. It is known that microbiota is an inducer of proinflammatory T helper 17 cells and regulatory T cells in the intestine. The signals at the sites of inflammation mediate recruitment and differentiation in order to remove inflammatory inducers and promote tissue homeostasis restoration. The change in the intestinal microbiota also influences the change in obesity and regresses the liver steatosis.

Evidence on the positive role of moderate alcohol consumption on heart and metabolic diseases as well on reducing steatosis have been looked up. Moreover nutrition as a therapeutic intervention in alcoholic liver disease has been discussed. In addition to the original data, we searched the literature (2008–2016) for the latest publication on the described subjects. In order to obtain the updated data we used the usual engines (Pub Med and Google Scholar).

The intention of the eighth symposia was to advance the international profile of the biological research on alcoholism. We also wish to further our mission of leading the forum to progress the science and practice of translational research in alcoholism.

Contents

1. Lieber's and his colleagues' legacy

Manuela G. Neuman M.Sc., Ph.D.

Ethanol is first metabolized in the liver to acetaldehyde (Lieber, 1997, 1988a). Also this metabolic pathway is present in the hepatocyte cytosol via a reaction catalyzed by the enzyme alcohol dehydrogenase (ADH) and this process may occur in the entire digestive tract leading to inflammation and chronic diseases. Subsequently the acetaldehyde is metabolized to acetate in the mitochondria being catalyzed by acetaldehyde dehydrogenase (ALDH). Like ADH, ALDH has multiple isoforms with differing activities in special populations (Sun et al., 2002).

Alcohol oxidation requires initial binding and reduction of the coenzyme nicotinamide-adenine dinucleotide (NADN). Mitochondrial NADN is oxidized through the electron transport chain by the specific enzyme NAD-dehydrogenase. Acetaldehyde also binds to macromolecules including nucleic acids, lipids and proteins, leading to autoimmune reactivity (Lewis and Zimmerman, 1998).

Lieber's biological research on alcohol-induced toxic effects led to the discovery of the cytochrome p450 (CYP) 2E1-dependent microsomal ethanol oxidizing system (MEOS) (Lieber and DeCarli, 1968, 1970). MEOS has been involved in alcohol-drug interactions (Lieber, 1988b; Lieber and De Carli, 1991), alcohol-induced fatty liver (Lieber et al., 1975) and non-alcoholic fatty liver disease (NAFLD) (Lieber, 2004). The diverse aspects of the damage include the character of the injury, the mechanism of the hepatotoxic effects, alcohol dose and frequency of exposure, and the medical and social importance (Lieber, 1978).

Epidemiological and experimental evidence has led to recognition of the key toxic role of alcohol in the pathogenesis of alcoholic liver disease (ALD) (Zimmerman, 1999). Also, the proven direct hepatotoxic effects of ethanol have undermined the observation that the hepatic disease of alcoholism is due to the contribution of malnutrition to the liver injury of alcoholism and evolution of alcoholic cirrhosis was defined (Zimmerman, 1955). The efficiency of alcohol as a substrate for energy production appears to be influenced by the amount of both alcohol and fat consumption as well as by gender (Falck-Ytter and McCullough, 2000).

Frenzer et al. (2002) also described the polymorphism in alcohol-metabolizing enzymes, glutathione S-transferases and apolipoprotein E that increases susceptibility to alcohol-induced cirrhosis and chronic pancreatitis.

ALD may coexist with other organ damage related to alcohol misuse, in the presence of therapeutics (Zimmerman and Maddrey, 1995). In addition alcohol can affect the pharmacokinetics of drugs by altering gastric emptying or liver metabolism. On the other hand therapeutics and or drugs of misuse may affect the pharmacokinetics of alcohol by altering gastric emptying and inhibiting gastric alcohol dehydrogenase (ADH), important in the first-pass metabolism (Lieber, 1988a). Castle and colleagues (2016) identify (2005–2011) the incidences of adverse drug reactions with alcohol involvement in the emergency departments of the Unites States of America and compared characteristics and disposition between these visits and visits of patients with adverse drug reactions without alcohol incidence. The visits involving alcohol-induced adverse drug reactions increased for males and females with ages 21 to 34 and females with ages over 55. Alcohol involvement increased odds of more serious outcomes from reactions. Central nervous system agents were the most common medications (59.1% mainly opioids and psychotherapeutic agents, including antidepressants; Neuman et al., 2006). There is a potential interaction between alcohol and H2 receptor antagonists such as cimetidine (Weinberg et al., 1998). The inhibition of the metabolism of acetaldehyde may cause disulfiram-like reactions. Pharmacodynamic interactions between alcohol and prescription drugs are common, particularly the additive sedative effects with benzodiazepines and also with some of the antihistamine drugs; other interactions may occur with tricyclic antidepressants.

1. Alcohol intake may be a contributing factor to the disease state which is being treated and may complicate treatment because of various pathophysiological effects (e.g. impairment of gluconeogenesis and the risk of hypoglycaemia with oral hypoglycaemic agents). The combination of nonsteroidal anti-inflammatory drugs and alcohol intake increases the risk of gastrointestinal haemorrhage (Zimmerman, 1999).

Moreover, Neuman et al. (1998) demonstrated the role of cytokines in ethanol-induced hepatocytotoxicity.

The purpose of the innovative research is to use advanced technologies to elucidate different aspects of alcohol-induced organ damage. Since 2009, we meet each year before the Research of Alcoholism annual meeting to celebrate new achievements in understanding the role of alcohol-induced organ injury.

2. Pathologic mechanisms of cell cycle arrest in alcoholic hepatitis

Samuel W. French, M.D.

Anna May Diehl focused first on the mechanism of regeneration inhibition of the liver in rats fed ethanol in response to partial hepatectomy (Koteish et al., 2002). The team reported that p21 and p27 were upregulated causing the inhibition of regeneration. Next, French et al. (2012) reported that p27 and p21 were upregulated in liver biopsies from patients with alcoholic hepatitis. Next, Aravinthan et al. (2013) reported that liver biopsies from two cohorts of alcoholic patients, (ALD and alcoholic cirrhosis) showed an increased expression of p21 in positive correlation with the degree of fibrosis. The p21 expression increased focally where the amount of fibrosis increased focally within the same liver. They showed that the pan cycle marker (Mcm-2) was upregulated in ALD but the S phase marker (Cyclin A) and the M phase marker (PH3) were downregulated, whereas p21 was markedly upregulated. Liver cell function was decreased, i.e. prothrombin time was increased in ALD and alcoholic cirrhosis and serum albumin levels were decreased in alcoholic cirrhosis. The levels of p21 correlated positively with the length of event free survival in both the ALD and cirrhosis cohorts. The Meld score and degree of alcohol consumption correlated to a lesser degree than the p21 levels. The p21 expression did not correlate with the grade of steatosis, steatohepatitis or bilirubin levels, the Meld Score or the amount of alcohol consumed at the time of the biopsy, in either cohort. There was an association between senescence measured by an increased hepatocyte p21 expression and impaired liver function. Increasing senescent liver cell change may have led to loss of function and liver cell mass, leading eventually to decompensation and death. This would explain progressive liver disease, since 81% of hepatocytes in the ALD cohort over expressed p21. p21 induced senescence is irreversible (Aravinthan et al., 2013).

A study of liver explants from patients with alcoholic hepatitis with Mallory-Denk bodies and balloon cell change showed virtual absence of the marker of regeneration, where only a few hepatocytic nuclei stained positive for Ki67. Instead the liver cells had changed into hepatic progenitor cells and bile ductules (bile duct metaplasia) (Dubuquoy et al., 2015).

In a study of liver biopsies from patients with alcoholic hepatitis where global RNA sequencing was performed, an increase in the expression of p21, p27 and p15 cell cycle inhibitors was found (Liu et al., 2015a, 2015b). p21 results in CKD inhibition and cell cycle arrest, preventing the replication of damaged DNA (Ko and Prives, 1996). p21 specifically inactivates G1 (CDK4 and 6). p21 also inhibits DNA synthesis by binding to and inhibiting proliferating cell nuclear antigen (PCNA). p21 is under transcriptional control of the p53 tumor suppressor gene.

p15 and 27 increase in response to transforming growth factor β (TGFβ). TGFβ was upregulated in alcoholic hepatitis when measured by RNA seq (Liu et al., 2015b) which contributes to growth arrest (Vermeulen et al., 2003). ATM was also upregulated. ATM phosphorylates p53 in response to DNA damage, resulting in p21 blocking the cell cycle at the G1/S checkpoint (Vermeulen et al., 2003).

P27 expression was upregulated in the alcoholic hepatitis liver biopsy study (Liu et al., 2015a, 2015b) in response to miR-34a expression upregulation. The miR-34a promoter contains p53 binding sites. p53 is a strong inhibitor of miR-34a. The mRNA level of p53 was downregulated. This suggests that miR-34a was upregulated because of the downregulation of p53 and upregulation of the expression of p27 by miR-34a (Liu et al., 2015b). p27 is a cell cycle inhibitor of GO/GI and GI/S and has been shown to be expressed in the nuclei, which stained positive in alcoholic hepatitis (Fig. 1).

p27, like p21, plays a dual role as a tumor suppressor and oncogene (Serres et al., 2012).

p27 is also an inhibitor of the G2/M phase of mitoses as well. p27 also prevents activation of GTPase RhoA regulating actin dynamics and promotes tumor cell migration and invasion. p27 expression is high in quiescent cells causing cytokinesis failure (Serres et al., 2012).

p15 (P15INK4B) is a member of the INK4 family of CDK inhibitors, which specifically inactivates GI CDI (CKD4 and 6). It prevents the activation of the CDK kinases by cyclin D. Like p27, p15 increases in response to transforming growth factor β (TGFβ) contributing to growth arrest (Vermeulen et al., 2003). p15, like p27 expression, is upregulated by TGFβ in alcoholic hepatitis (Liu et al., 2015b).

ATM (ataxia-telangiectasis-mutated) and ATR (ataxia and rad3 related) recognize 5DNA damage and phosphorylate the p53 response to DNA damage at GI and G2 of the cycle. ATM is upregulated in alcoholic hepatitis (Liu et al., 2015a). They both respond to DNA damage by phosphorylating the downstream checkpoint kinases, Chk2 and Chk1, to transduce the damage signal (Elledge, 2015) and phosphorylate NSB1 to cause S phase arrest.

Transforming growth factor β (TGFβ) inhibits cell proliferation by inducing G1 phase cell cycle arrest. TGFβ induces p15 and p27.

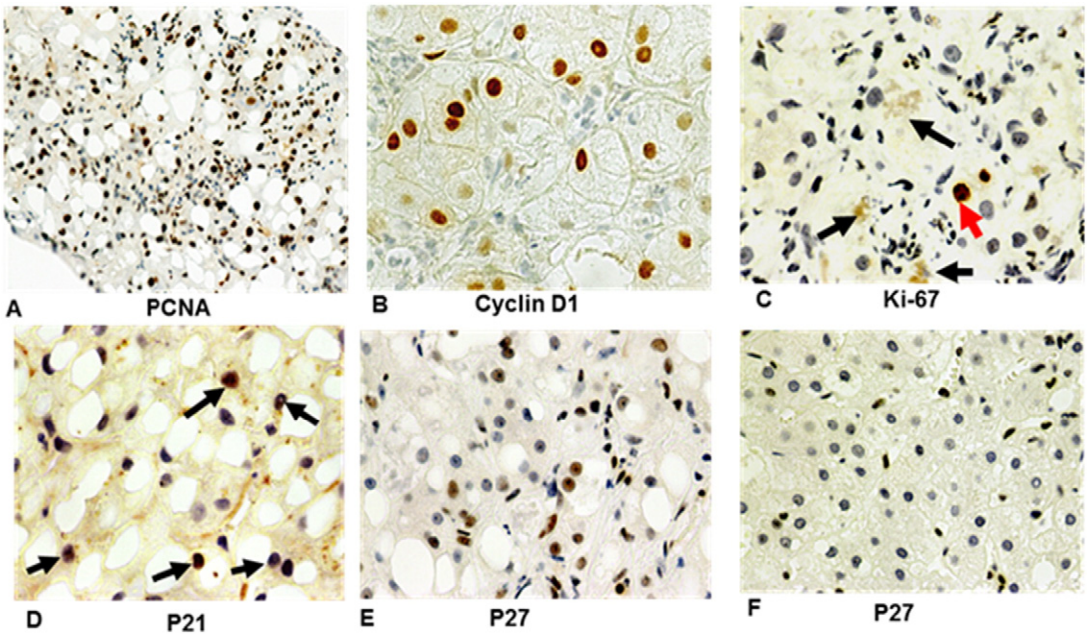

Fig. 1. The liver biopsy slides from patients with alcoholic hepatitis were stained with antibodies to PCNA (A), Cyclin D1 (B), Ki-67 (C), p21 (D) and p27 (E). Only a few scattered nuclei were positive for Ki67 (arrow). In one of the alcoholic hepatitis biopsies no nuclei stained positive. (F) The arrows point to Mallory Denk bodies staining yellow orange (C). Magnification (A × 218), (B × 654), (C × 654), (D × 684), (E × 436), (F × 436).
This figure was previously published in Exp. Mol. Pathol. 92: 318–326 (2012).

Activation and expression of p15 and p27 is upregulated by TGFβ (Vermeulen et al., 2003). TGFβ expression is upregulated in alcoholic hepatitis (Liu et al., 2015b).

It is concluded that alcoholic hepatitis inhibits liver cell regeneration creating senescent hepatocytes through a variety of mechanisms including induction of numerous cell cycle inhibitors i.e. p21, p27, p15, ATM and TGFβ (Fig. 2).

3. Mendelian randomization in alcohol research

Samir Zakhari, Ph.D.

For over 30 years, countless epidemiological and molecular studies have pointed at potential benefits of moderate drinking, including reduction in risk of coronary artery disease and all-cause mortality, among others. While the definition of moderate drinking varies

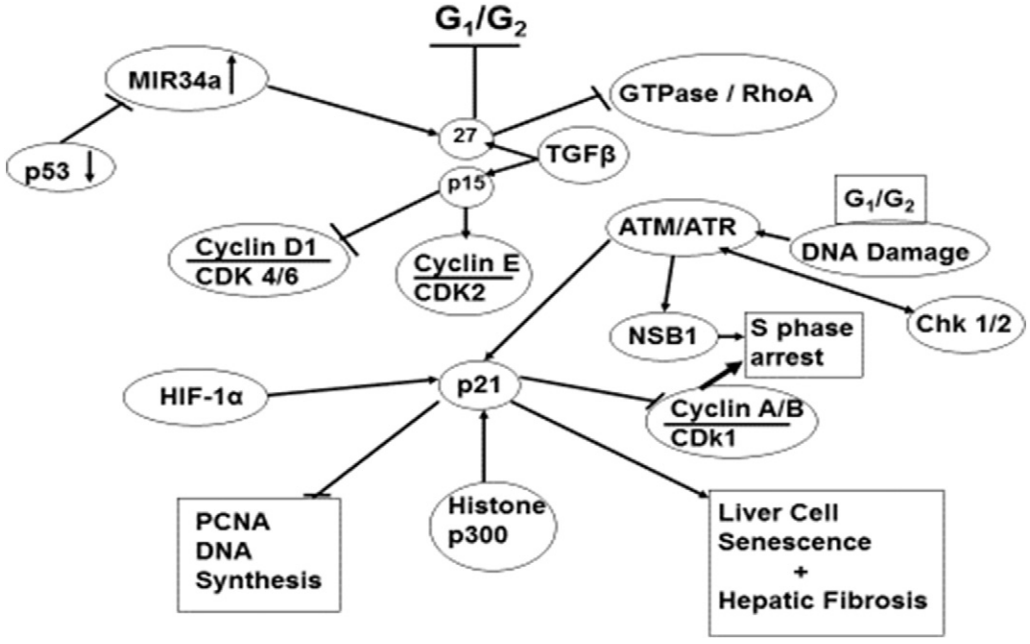

Fig. 2. Schematic overview summarizing the inhibition of the cell cycle found in alcoholic liver biopsies from patients with alcoholic hepatitis.
The diagram is a modification from Fig. 2 in the paper by Vermeulen et al. (2003) This figure was previously published in Exp Mol Pathol 100: 502–505 (2016).

between countries (Furtwængler and De Visser, 2013), the United States Dietary Guidelines (https://health.gov/dietaryguidelines/2015/) defines moderate drinking as up to one drink per day for women and up to two drinks per day for men — and only by adults of legal drinking age. One alcoholic drink-equivalent is described as containing 14 g (0.6 fl oz) of pure alcohol; for reference one alcoholic drink-equivalent comprises 12 fluid ounces of regular beer (5% alcohol), 5 fluid ounces of wine (12% alcohol), or 1.5 fluid ounces of 80 proof distilled spirits (40% alcohol) (http://www.ars.usda.gov/nea/bhnrc/fsrg). As early as 1996, Rimm and colleagues have concluded that a substantial portion of the decreased risk of coronary artery disease is attributed to alcohol rather than to other components of alcoholic beverages (Rimm et al., 1996).

While observational epidemiological studies can help identify disease incidence in a community, they are by necessity associative and cannot determine cause and effect relationships (Zakhari and Hoek, 2015). Despite best efforts to improve design and analysis of observational studies, some correctly stated that "Proof is impossible in epidemiology" (Conner, 2016). This is primarily due to the limited number of confounders measured in epidemiological studies, and the inevitable measurement errors in assessing both the exposure and the potential confounders (Phillips and Smith, 1991, 1993).The problem is more accentuated in alcohol epidemiological studies because all these studies rely on self-reporting to determine the amount and type of alcoholic beverage consumed, which inevitably introduces recall bias (Klatsky et al., 2014). As early as 1965, Hill (Hill, 1965) observed that for epidemiological observation to infer causation, several criteria should apply, including: strength of association, consistency, specificity, among others. The causal link between exposure to a given factor and disease is of public health concern, as it illuminates the way to prevention and treatment measures. While observational epidemiology contributed to the causal discovery of exposure and disease (e.g., asbestos and mesothelioma, smoking and lung cancer, and ZIKV infection and microcephaly and neurological complications (Rasmussen et al., 2016), alcohol observational studies fall short of proving cause and effect. Thus, coupling epidemiological studies with molecular and genetic ones would strengthen the cause and effect relationships. Thus, genetic epidemiology can contribute to illuminating cause and effect in health sciences, and ultimately to a comprehensive molecular understanding of pathogenesis (Khoury and Dorman, 1998). Genetic epidemiology focuses on the association between genetic and phenotypic variation within a population to elucidate the genetic basis of disease, often based on single nucleotide polymorphisms (SNPs). The random assignment of genes within populations is also used to reduce confounding in examining exposure–disease associations, which is known as "Mendelian Randomization (MR)." This approach was successfully used to examine environmental exposure and coronary heart disease causation" (Youngman et al., 2000; Keavney et al., 2006). MR is based on the premise that the distribution of genetic variants in a population is independent of environmental and behavioral factors that confound epidemiological associations between exposure and disease. Thus, polymorphisms with a well-characterized biological function can be utilized to study the effect of a given exposure on disease risk. This approach avoids several potential problems, such as confounding, reverse causation (biological or due to reporting bias), associative selection bias, or attenuation by errors in observational epidemiology. (Smith and Ebrahim, 2004) In other words, the association between a disease and a polymorphism that serves as a proxy for exposure is not generally susceptible to confounding factors or to reverse causation that may distort the interpretations of observational studies. However, the limitations for the use of MR in epidemiological studies include: 1) a robust association between genetic variants and health outcomes must be established, which so far has proven to be difficult (Colhoun et al., 2003); 2) any association between a genetic variant and health outcome may be confounded by linkage disequilibrium between the variant under consideration and another variant influencing disease risk; 3) if the variant has pleiotropic effects, interpretation of any associations between a genetic variant and health outcomes may not be simple (Smith and Ebrahim, 2004).

In Fig. 3 we designed how the MR can be wrongly used.

3.1. Alcohol and coronary heart disease

Numerous studies, molecular and epidemiological, about the possible protective effect of moderate alcohol consumption on CHD risk have been published, e.g., (Klatsky, 2001). Mechanisms contributing to the decrease in CHD risk associated with moderate drinking include an increase in the levels of the protective high density lipoprotein (HDL) cholesterol (Rimm, 2001; Zakhari and Gordis, 1999).

Mendelian randomization studies are valuable when functional polymorphisms exist, such as polymorphisms in genes producing alcohol metabolizing enzymes. Oxidative alcohol metabolism is carried out mainly in the liver, where ethanol (alcohol) is metabolized to acetaldehyde by alcohol dehydrogenase (ADH) in the cytosol to produce acetaldehyde, which is further metabolized to acetate by mitochondrial aldehyde dehydrogenase (ALDH2) (Zakhari, 2006). Polymorphisms exist in *ADH1B*, *ADH1C*, and *ALDH2*. Two polymorphic forms of *ADH1C* (*ADH1C*1* and *ADH1C*2*) produce two different enzyme subunits, γ1 and γ2, which metabolize alcohol in a fast and slow manner, respectively (Fig. 3). Thus, individuals with the slow oxidizing enzyme, who clear alcohol at a slower rate, are expected to have a lower risk of coronary artery disease. Indeed, this was the finding in a case-control study. The risk ratios in heterozygote (γ1γ2) and homozygous (γ2γ2) was 0.90 and 0.72, respectively compared to homozygous fast alcohol oxidizers (γ1γ1) (Hines et al., 2001). In addition, γ1γ1 carriers had lower HDL cholesterol levels than the γ2γ2 slow oxidizers. Thus, the biological

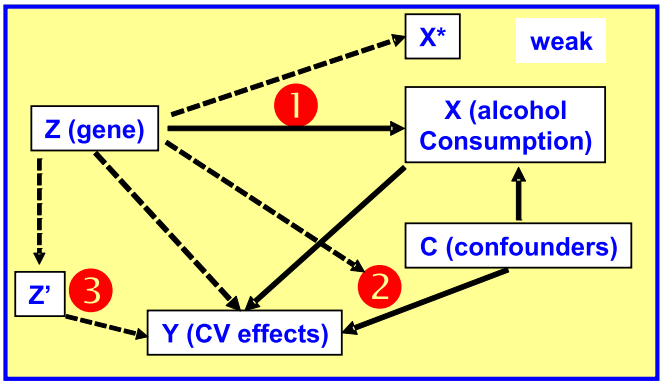

Fig. 3. Mendelian Randomization assumptions violated.

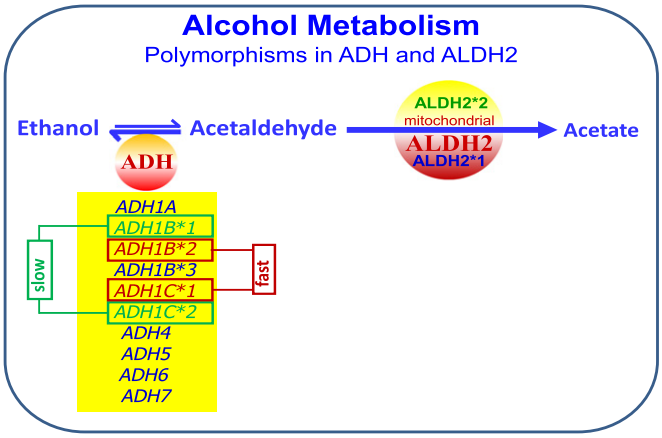

Fig. 4. Polymorphism in *ADH* and *ALDH* genes.

Table 1
Genetic instrument selection.
Modified from Vu et al. (2016).

Genes	rs number	References	LD lipid loci (r^2)	Correlation with confounders (r)	Final instruments
ADH1B	rs1229984	Gelernter et al. (2014), Zuccolo et al. (2009), Agrawal and Bierut (2012), Ferrari et al. (2012), Li et al. (2011), Bierut et al. (2012), Way et al. (2016)			Did not pass quality control
ADH1B	rs2066702	Zuccolo et al. (2009)	0.003	0.011	Yes
ADH1B	rs1693457	Zuccolo et al. (2009)	0.001	0.015	Yes
ADH1B/1C	rs1789891	Agrawal et al. (2012)	0.001	0.023	Yes
ADH1C	rs1693482	Gelernter et al. (2014), Ferrari et al. (2012), Agrawal et al. (2012), Toth et al. (2011)	0.000	0.011	No, in high LD with rs698 and has lower sample size
ADH1C	rs698	Ferrari et al. (2012), Bierut et al. (2012), Agrawal et al. (2012)	0.001	0.015	Yes
ADH1C	rs1614972	Zuccolo et al. (2009), Ferrari et al. (2012)			Violation HWE assumptions

effect of these variants is equivalent to moderate alcohol intake. These findings do not necessarily infer that only people with the slow genotype will benefit from moderate drinking, rather the whole population (regardless of their genotype) would benefit (Smith and Ebrahim, 2003). While there was no strong association of these polymorphic variants and alcohol intake, the variant of *ALDH2* (*ALDH2*2*) which is virtually inactive and is prevalent in East Asians is associated with facial and nausea in response to drinking, resulting in reduced alcohol consumption and protection against alcoholism (Nakamura et al., 2002).

3.3. Polymorphisms in ADH1B, ADH1C, ALDH2

Alcohol dehydrogenase polymorphism is graphically represented in Fig. 4.

The Atherosclerosis Risk in Communities (ARIC) study used a Mendelian Randomization (MR) approach to examine whether alcohol consumption causally affects lipid profile (Vu et al., 2016). Their findings using over 10,000 subjects support the causal role of regular low-to-moderate alcohol consumption in increasing high density lipoprotein (HDL)2-c, reducing total cholesterol, and low density lipoprotein (LDL)-c, and provides evidence for the novel finding of reducing apoB and sdLDL-c levels among European Americans. While data in this study is based on self-reported alcohol consumption, and the MR was used to reduce reverse causation, sensitivity analysis was conducted that excluded never drinkers and heavy drinkers. The effect of alcohol consumption on those lipids remained significant after excluding heavy drinkers. As shown in Table 1, these SNPs were evaluated by instrumental variable (IV) analysis which resulted in rs 1229984, rs 1693482, and rs1614972 removal because they did not meet the IV assumptions, and possible violations including linkage disequilibrium. The fact that the score was calculated from different genes further strengthens the causal inference. A study on a Chinese population using the MR approach showed that the minor *ALDH2* allele rs 671was associated with a reduction in alcohol consumption and HDL cholesterol (Taylor et al., 2015). In addition, Zhang et al. (2015) on over 4800 Chinese men found that *ALDH2* genotype is strongly associated with alcohol use, but not with alcohol-related health outcomes. However, using *ALDH2* as a genetic instrument in MR studies could be problematic because the ALDH2 activity can change by aging or medication, and thus may influence alcohol use and associated diseases (Chen et al., 2013). Holmes et al. (2014) using MR to study the association between alcohol consumption and cardiovascular disease compared alcohol intake and ischemic heart disease in carriers vs. non-carriers of the *ADH1B* rs1229984 allele which encodes the ADH1B enzyme and have concluded that "reduction of alcohol consumption, even for light to moderate drinkers, is beneficial for cardiovascular health". This study was criticized on the British Medical Journal (http://www.bmj.com/content/349/bmj.g4164/rapid-responses). Roerecke and Rehm (2015) advise the need for closer examination to ensure the rules of MR.

a) The gene (Z), in this case, rs1229984, must be related to alcohol intake (X). This condition is met because carriers of the A allele drank fewer alcohol units. However, the association is weak since it is based on self-report (X*).
b) rs1229984 must be unrelated to confounders (C) of the alcohol-CVD response. Results of this study indicate that this gene has other (non-alcohol-related) effects on CVD such as blood pressure, body mass index, inflammatory markers, and lipids, which confounds the outcome. In other words, the effect could be due to these risk factors unrelated to alcohol consumption.
c) There should be no direct causal association between rs1229984 and CVD (Y) that does not go through alcohol use. However, another SNP (or SNPs) (Z′) may be in linkage disequilibrium with ADH1B and provide direct causal relation with CVD.

The study also assumes that the *ADH1B* genotype ONLY influences drinking amount. That is highly likely to be wrong since ADH1B metabolizes many other compounds, some of which could also affect the outcome. It also assumes that the *ADH1B* genotype is evenly distributed among ethnic groups. The allocation of the A-allele variants is far from random, which introduces an entire new set of confounds (such as many subtle differences in minor allele frequency with many socio-economic and behavioral differences). In fact the study showed low prevalence of the rs1229984 A-allele (average carriage: 7%). Furthermore, 41 out of the 56 studies used (corresponding to 84% of participants) had a proportion of A-allele carriers less than 10%.

Finally, Roerecke and Rehm (2015) stated that there is not enough power "to investigate these limitations thoroughly because allele carriers are rare in many European countries." New and novel epidemiological studies with better design, including but not limited to MR would go a long way in determining causality between alcohol consumption and health effects.

4. Microbiome and non-alcoholic fatty liver disease

Stephen Malnick M.D.

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome (Yu et al., 2016). It is a major public health issue and is a leading cause of cirrhosis, its complications including hepatocellular carcinoma and the need for liver transplantation. The pathogenesis of NAFLD results in inflammation (steatohepatitis) and fibrosis.

The human gut microbiome consists of about 1014 bacterial cells, which include >200 species of anaerobic bacteria (Neish, n.d.). This is 100 times more genes than in the human genome (Bäckhed et al., 2004).

There is an interaction between the liver and the fecal microbiome (Nicholson et al., 2012). The liver receives 70% of its blood supply from the intestine via the portal vein (Manzano-Robleda et al., 2015).

Thus it is to be expected that there will be an interaction between the gut microbiome and the liver. Bile acids have been shown to facilitate the communication between the intestine and the liver (Dawson and Karpen, 2015). They regulate hepatic glucose, lipid metabolism and inflammation via the farsenoid X receptor (FXR) (Fuchs et al., 2013; Pineda Torra et al., 2003).

There appears to be other factors involved in the development of NAFLD than the FXR. The *ob*/*ob* FXR knockout mice have been shown to have improved glucose homeostasis including increased glucose clearance and adipose tissue insulin sensitivity, but hepatic triglyceride content increased and hepatic insulin sensitivity was unchanged (Prawitt et al., 2011). This paradoxical effect may be related to the microbiome. The gut microbiota can impact on the pathogenesis of NAFLD via several mechanisms.

4.1. Obesity

Obesity is an essential component of the metabolic syndrome. The gut microbiota has been shown to be an environmental factor that regulates fat storage. Germ free mice have been shown to gain 42% less weight compared to mice that acquired a microbiome at birth (Bäckhed et al., 2004). This was despite the fact that they consumed 29% less chow. Furthermore the intestinal microbiota has been shown to determine the development of NAFLD in mice. C57BL/6J mice fed a high fat diet may respond by developing hyperglycemia, hepatic inflammation and steatosis. When germ-free mice are colonized with microbiota from such responder mice, there is a transfer of insulin resistance and increased hepatic steatosis as compared to the mice colonized with microbiota from non-responder mice (Le Roy and Llopis, 2013). Other evidence implicating the fecal microbiota in the development of obesity include mice receiving microbiota from obese donors having a higher fat gain compared to those receiving from lean donors (Turnbaugh et al., 2007), and fecal short chain fatty acid levels are 20% higher in obese humans compared to lean volunteers (Vrieze et al., 2008; Schwiertz et al., 2010). One of the key recommendations for treating NAFLD is exercise. Exercise has recently been shown to have an impact on gut microbial diversity. A group of 40 elite rugby players from Ireland were shown to have a larger microbial diversity than control groups with a BMI of <25 kg/m^2 or >28 kg/m^2 (Clarke et al., 2014).

There has been a marked increase in the prevalence of both obesity and NAFLD in the last 2 decades. There may be a role for the increased use of artificial sweeteners in this trend. Mice fed a high fat diet and also given saccharin in the drinking water have a higher level of serum glucose after a glucose load compared to mice receiving glucose in their drinking water. This difference was weakened after antibiotic administration (Suez et al., 2014). Furthermore in human volunteers consuming the recommended daily dose of artificial sweeteners, there was a larger increase in serum glucose after an oral glucose load. In addition a nutritional survey found that those who consumed a high amount of artificial sweeteners had a significantly higher HBA1c level than those who did not. Finally this group also found that the microbial diversity was higher in those responding to an oral glucose test after consuming a high amount of artificial sweetener for a week compared to those who did not respond. There may also be a role for bacteria in protecting from obesity. *Akkermansia municiphila* has been shown to be protective against insulin resistance in humans and to be associated with smaller sized adipocytes (Dao et al., 2016).

4.2. Ethanol

Ethanol has been shown to have many effects on the gut and liver. It reaches the liver via the portal vein, induces triglyceride accumulation in the liver together with hepatic oxidative stress (Sarkola and Eriksson, 2001) and also increases the gut permeability. Serum ethanol levels have been found to be higher in patients with NASH compared to both non-obese and obese patients without NASH (Zhu et al., 2013).

There is, however, controversy over whether consumption of a small or moderate amount of alcohol is beneficial for patients with NASH (Seitz et al., 2015; Sookoian and Pirola, 2016; Roerecke et al., 2016a, b).

Endotoxin is part of the gram negative bacterial cell membrane. Lipopolysaccharide (LPS) is the active component of endotoxin and interacts with Toll-like receptors to start an inflammatory cascade (Ruiz et al., 2007). Genetically obese mice have been shown to develop steatohepatitis after infusion of low doses of LPS (Yang et al., 1997). In addition in humans higher endotoxin levels have been associated with NAFLD (Harte et al., 2010).

Inflammasomes are cytoplasmic multi-protein complexes and sensors of pathogen-associated molecular patterns (PAMPS). Mice with deficient inflammasome activation have been shown to have increased NASH severity and furthermore this increased severity can be transferred to wild-type mice via transfer of microbiota (Henao-Mejia et al., 2012). Toll-like receptor-4 (TLR-4)-chimeric mice treated with LPS challenge have been shown to promote hepatic fibrosis by stellate cell activation (Seki et al., 2007). Thus endotoxins from the bacterial microbiome promote hepatic inflammation and fibrosis which can promote the development of NASH and cirrhosis. Choline is an important

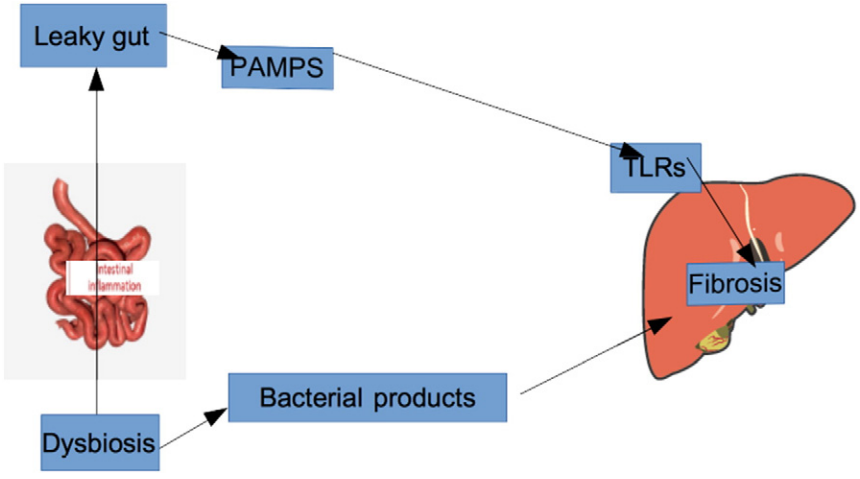

Fig. 5. The link between microbiome, dysbiosis and fibrosis.

phospholipid in cell membranes and gut microbiota produce enzymes that catalyze choline into methylamines which can cause inflammation in the liver (Zeisel et al., 1983). Patients who receive total parenteral nutrition can develop steatosis related to choline deficiency and this is prevented by choline replacement (Buchman et al., 2001). Furthermore the gut microbiome has been shown to change in choline-deficient patients associated with changes in liver fat (Spencer et al., 2011).

Gut dysbiosis refers to disruption of the normal gut microbiota it is present in obesity and NAFLD. A high prevalence of small intestinal bacterial overgrowth (SIBO) has been found in obese patients undergoing bariatric surgery. 137 patients underwent a hydrogen breath test and 136 had an intraoperative liver biopsy (Sabaté et al., 2008). SIBO was shown to be an independent risk factor for the presence of severe steatosis with an odds ratio of 27.5. Furthermore dysbiosis is related to NAFLD. A study comparing 53 NAFLD patients with 32 healthy controls showed a significant difference in the genus composition between the two groups (Jiang et al., 2015).

Furthermore, there was an increase in the size of the tight junctions in the duodenal mucosa and a decrease in the amount of occludin in the mucosal cells. Occludin is the structural backbone of the tight junctions. In addition there is an increase in toll-like receptor signaling linked to SIBO in patients with NAFLD (Kapil et al., 2016). Recently, it has been shown that there is a link between gut dysbiosis, the severity of NAFLD and a shift in the metabolic function of the gut microbiome (Boursier et al., 2016). There was a decrease in *Prevotella* and an increase in *Bacteroides* when comparing both patients with NASH and without NASH and also when comparing patients with NASH and minor fibrosis to those with more advanced fibrosis. In addition, when using a technique termed PICRUST for examining the metagenomic profile, it was found that there was an increase in bacteria employing the KEGG pathway involving metabolism of carbohydrates, lipids and amino acids.

It seems that the microbiome may contribute to liver disease in several ways as illustrated in the Fig. 5.

It may be possible to modulate the microbiome in order to treat NAFLD. One of the central features of the metabolic syndrome is obesity and weight loss is an important component of any treatment regimen for NAFLD. Weight loss has been shown to produce changes in the fecal microbiome, both in terms of metabolic products and bacterial communities (Patrone et al., 2016). In a small study, transfer of intestinal microbiota from a lean donor was found 6 weeks later to increase insulin sensitivity in treatment-naive patients with the metabolic syndrome (Vrieze et al., 2012a, 2012b). Unfortunately lifestyle changes alone are not sufficient in most patients and further intervention is required. Administration of prebiotics to patients with NAFLD has been examined. A meta-analysis, although showing a decrease in serum transaminases, did not show a significant change in either the level of TNF-alpha or the HOMA index (Ma et al., 2013). It is difficult to draw conclusions about the effects of probiotics since animal models and bacterial strains are different, the gut microbiota will always outnumber the probiotics that can be administered and there is a variability of the human microbiome, diet and genetics.

Probiotics are non-digestible plant-derived carbohydrates that act as a fermentation substrate within the colon. They can stimulate the preferential growth and activity of a limited number of microbial species that confer health benefits on the host. A meta-analysis of 26 randomized controlled trials of prebiotics in humans showed a beneficial effect on post-prandial glucose and insulin concentrations (Kellow et al., 2014). This may have a beneficial effect on NAFLD but remains to be determined.

Together with my colleagues we are investigating the effects of fecal transplantation from a thin donor on 10 obese patients and 10 control patients undergoing a screening colonoscopy. The results are eagerly awaited. However, it has recently been estimated that in order to have the power to detect a beneficial effect on obesity hundreds of patients will need to be included in the studies going forward (Falcony et al., 2016).

In summary, there is an effect of the fecal microbiome on both the development and progression of NAFLD. Studies on the therapeutic implications of this finding are just beginning.

5. The role of dietary fat in the gut-liver axis in alcoholic liver disease

Irina A. Kirpich, M.P.H., Ph.D

Diet and crosstalk between the gut and the liver are important determinants of alcoholic liver disease (ALD) (Kirpich et al., 2016a, 2016b). Numerous studies, have shown that dietary unsaturated fat (specifically omega 6 lipids) exacerbates alcohol-mediated intestinal permeability, liver steatosis, inflammation, and injury (Nanji and French, 1989; Kirpich et al., 2012, 2013; Chen et al., 2015; Zhong et al., 2013; Ronis et al., 2004). These pathological effects were prevented/blunted by dietary saturated fat, suggesting a significant contribution of specific dietary lipids in ALD development and progression. As shown in a number of recent clinical (Gabbard et al., 2016; Bode et al., 1993, Morencos et al., 1995, Tuomisto et al., 2014) and preclinical studies (Bull-Otterson et al., 2013, Mutlu et al., 2009, Yan et al., 2011), alcohol intake and alcohol-induced liver injury are associated with qualitative and quantitative alterations of gut microbiota. We have recently demonstrated that dietary saturated fat (SF), rich in medium chain triglycerides [MCT] and beef tallow and unsaturated fat (USF, rich in corn oil) differentially modulate gut microbiome, intestinal barrier and liver injury in a mouse model of ALD (Kirpich et al., 2016a, 2016b). Thus, compared to SF + EtOH, USF + EtOH administration produced hepatic steatosis, inflammation, and injury. In parallel with liver injury, significantly elevated serum LPS levels, intestinal inflammation and increased gut permeability with intestinal tight junction and mucus layer alterations were observed in mice fed USF + EtOH but not SF + EtOH. Major alterations in gut microbiota, including a prominent reduction in *Bacteroidetes*, and an increase in *Proteobacteria* and *Actinobacteria*, were seen in USF + EtOH but not in SF + EtOH fed animals, suggesting that the types of dietary fat play a critical role in ethanol-mediated changes of the composition of the gut microbiota. The increase in *Proteobacteria* phylum provides a possible link between the alterations of the gut microbiota and hepatic inflammation via endotoxin, a component of the Gram negative bacteria outer membrane. It has been shown that unlike dietary SF, USF feeding promoted ethanol-mediated reduction of commensal bacteria (e.g., Lactobacillus species) that produce beneficial factors for maintaining barrier function in intestinal epithelial cells. Characterization of both microbiota composition and function is an important approach to investigate host–microbial interaction.

In comparison to SF + EtOH, USF + EtOH caused major fecal metabolomic changes, including significant reduction in numerous long- (hexadecanoic and heptadecanoic), medium- (hexanoic and octanoic), and short- (butanoic) free fatty acids. A decline in certain fecal amino acids (e.g. serine and glycine) was also observed in USF + EtOH fed animals. Remarkably, the levels of octanoic acid, which possesses some antimicrobial properties, were dramatically

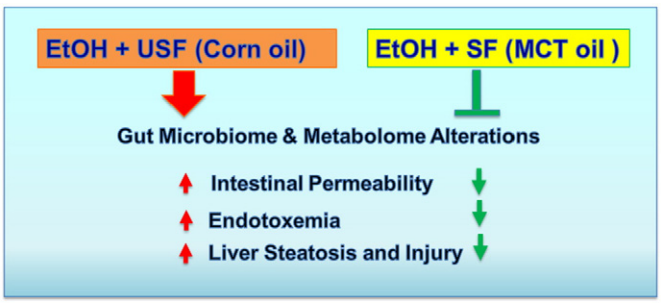

Fig. 6. Dietary saturated and unsaturated fats differentially modulate gut microbiome, metabolome, intestinal barrier and liver injury in a mouse model of alcoholic liver disease. Abbreviations: EtOH, ethanol; MCT, medium chain triglycerides; SF, saturated fat; USF, unsaturated fat.

reduced (~50 fold) in mice fed USF + EtOH compared to SF + EtOH fed animals. The relative deficiency of octanoic acid may contribute to the expansion of certain types of bacteria and overall changes in the gut microbial population caused by USF + EtOH. The protective effects of SF diet on ethanol-mediated changes of the gut microbiota might be also attributed to octanoic acid, as it was the major fatty acid in SF diet. Further studies will be needed to test these hypotheses. Given that short free fatty acids play an important role in the intestinal and host health, the decrease in fecal butanoic acid may contribute to the intestinal inflammation and gut barrier disruption observed in USF + EtOH fed mice. In addition, butyrate possesses anti-inflammatory properties (Inan et al., 2000), and serves as a potent histone deacetylase inhibitor (Davie, 2009). In experimental ALD, butyrate supplementation protected against alcohol-mediated intestinal tight junction disruption and liver inflammation (Cresci et al., 2014).

In summary, there is an increasing body of evidence demonstrating that different types of dietary fat differentially modulate susceptibility to EtOH-mediated alterations in the gut and the liver (Fig. 6), and suggest that the ethanol-mediated liver injury and gut dysbiosis can be therapeutically targeted by dietary manipulations (e.g., modulation of dietary lipids) that may offer a novel prevention/therapeutic approach in the ALD management.

6. Autophagy in alcohol-induced liver injury

Paul G. Thomes Ph.D., Laura W. Schrum Ph.D., Terrence M. Donohue, Jr. Ph.D.

The hallmarks of liver pathology that occur after years of heavy drinking include accumulation of lipid droplets, damaged proteins and defective organelles, which cause cellular toxicity, and ultimately, hepatocyte death (Donohue, 2009; Ji, 2015; Dolganiuc et al., 2012). Accumulation of toxic molecules in the liver can be partially attributed to dysfunction of intracellular degradation pathways, which tightly regulate turnover rates of proteins and clear the cell of obsolete macromolecules (Donohue, 2009). The two most important intracellular protein degrading systems in mammalian cells that maintain cellular homeostasis are the ubiquitin-proteasome system and lysosome-dependent autophagy (Donohue and Thomes, 2014).

Macro-autophagy (hereafter, called autophagy) is a process of intracellular degradation of a cell's own contents (i.e., "self-eating"). Autophagy degrades macromolecules (proteins, nucleic acids, carbohydrates, triglycerides) and dysfunctional organelles to generate pre-cursors for energy production, anabolic processes and to eliminate potentially toxic cellular waste (Moreau et al., 2010; Donohue, 2009; Donohue and Thomes, 2014). Cells activate autophagy in response to nutrient deprivation, oxidant stress and hypoxia (Donohue, 2009). Conversely, autophagy is suppressed by growth factors, nutrients and by nutrient sensing pathways (Neufeld, 2012). Autophagy begins within the cytoplasm, with the formation of a double membrane structure that sequesters substrates destined for degradation in a vesicle called autophagosome/autophagic vacuole (AV). The AV is trafficked to and fuses with a lysosome where its contents are degraded by lysosomal hydrolases. This process is regulated by the coordinated actions of autophagy-related gene products (Atgs) (Itakura and Mizushima, 2010). For more details of the autophagy pathway, please refer to the following review articles (Dolganiuc et al., 2012; Donohue and Thomes, 2014).

Early work in the lab of Dr. Donohue revealed that chronic ethanol exposure to rodents impairs hepatic lysosomal function (Donohue et al., 1989, 1994; Kharbanda et al., 1995), indicating that alcohol disrupts autophagy, as this pathway is dependent on lysosomes for macromolecular degradation (Donohue, 2009). Since disruption of autophagy is associated with a variety of liver diseases (Czaja et al., 2013) and such disruption of autophagy by alcohol could be the possible mechanism behind accumulation of toxic substances in the liver leading to alcoholic liver injury, we investigated how ethanol oxidation regulates autophagy in ethanol metabolizing hepatoma cells in vitro and in the livers of chronic ethanol-fed mice in vivo.

We measured autophagy by quantifying the AV marker protein LC3-II by immunohistochemistry and Western blot in ethanol non-metabolizing HepG2 cells and in recombinant VL-17A cells that metabolize ethanol through stably expressed alcohol dehydrogenase (ADH1) and cytochrome P4502E1 (CY2E1), after 50 mM ethanol exposure for 24 h (Thomes et al., 2013). Immunohistochemistry and Western blot analyses confirmed that ethanol exposure induced AVs only in VL-17A cells, which metabolized ethanol, as judged by acetaldehyde (Ach) production in the culture media (Thomes et al., 2013). Further, when we co-incubated VL-17A cells with ethanol and 4-methylpyrazole to block ethanol metabolism, VL-17A cells exhibited no AV induction, suggesting that ethanol oxidation is necessary for enhanced AV formation (Thomes et al., 2013). To further understand the temporal regulation of AVs by ethanol, we performed LC3-II flux measurements in the presence and absence of lysosomal inhibitor bafilomycin, to determine the rate of AV synthesis and their degradation. Bafilomycin blocks lysosomal degradation of substrates, including AVs by increasing lysosomal pH. Thus, elevated LC3-II levels in VL-17A cells co-incubated with ethanol and bafilomycin compared with cells exposed to ethanol or bafilomycin alone indicated that ethanol exposure enhanced the synthesis of AVs (Thomes et al., 2013). When we measured the levels of P62, an adaptor protein whose levels decrease during activation of autophagy, we found that ethanol exposure simultaneously increased P62 protein, suggesting that AVs accumulated in VL-17A cells due to enhanced synthesis and decreased degradation (Thomes et al., 2013). To validate whether ADH catalysis, which produces acetaldehyde (Ach) and CYP2E1 catalysis which predominantly generates ROS, have different effects on AV formation, we tested ethanol effects on LC3-II in VA-13 and E-47 cells, which metabolize ethanol through stably expressed ADH1 and CYP2E1, respectively. Interestingly, only VA-13 cells exhibited enhanced AV formation after ethanol exposure (Thomes et al., 2013). Further investigations revealed that HepG2 and E-47 cells did not produce Ach but VA-13 and VL-17A cells each produced Ach after ethanol exposure, indicating that the primary ethanol metabolite Ach is likely responsible for ethanol-induced disruption of autophagy (Thomes et al., 2013). This was supported by other findings that it is not exposure of acetate, a product of Ach metabolism, but rather exposure to Ach that induced LC3-II levels in VA-13 and VL-17A cells (Thomes et al., 2013). We confirmed the effects of Ach on autophagy when direct exposure of Ach (300 μM) for 24 h enhanced LC3-II protein in ethanol non-metabolizing HepG2 cells (Thomes et al., 2013). These findings suggest that the primary ethanol metabolite Ach, which is deemed responsible for much of the pathology associated with ethanol, disrupted autophagy in ethanol metabolizing HepG2 cells.

We extended our investigations on autophagy in vivo to livers of GFP-LC3 mice (C57/BL6) pair-fed the Lieber-DeCarli control or ethanol diet for six to eight weeks. GFP-LC3 mice are transgenic for the fusion protein green fluorescent protein-microtubule associated protein light chain 3 (GFP-LC3), thus AVs are readily visualized as fluorescent green puncta (dots) under the fluorescent microscope. Our microscopic analyses revealed that hepatocytes isolated from ethanol-fed mice exhibited higher levels of AVs than those from pair-fed control mice (Thomes et al., 2015), supporting our in vitro findings that ethanol metabolism induces AV formation. Ethanol feeding not only increased AV numbers but they also increased their average volume, suggesting that undegraded AV cargo was accumulating in these vesicles (Thomes et al., 2015). After we stained the lysosomes (Lys) and co-localized AVs with Lys, as an index of AV-Lys fusion, a crucial step in the degradation phase of autophagy, we found that hepatocytes from ethanol-fed mice exhibited fewer lysosomes and a lower frequency of AV-Lys co-localization compared with hepatocytes from pair-fed control mice (Thomes et al., 2015). We reported similar findings in ethanol exposed VL-17A cells (Thomes et al.,

2013). These findings reveal that ethanol caused defects in AV-Lys fusion. We verified these findings using immunohistochemical staining in crude liver homogenates and in isolated lysosomal fractions of livers. Ethanol-fed mice exhibited enhanced LC3-II levels compared with mice fed the control diet (Thomes et al., 2015). In these same fractions we simultaneously detected higher P62 levels in livers of ethanol fed mice than in pair-fed control mice (Thomes et al., 2015), supporting our in vitro findings that ethanol exposure enhanced AV synthesis, but it simultaneously decreased AV degradation. When we quantified free GFP derived from GFP-LC3 hydrolysis as another index of autophagy flux, we detected lower levels of free GFP in ethanol-fed mice livers than in liver homogenates of pair-fed control mice (Thomes et al., 2015), confirming that chronic ethanol exposure slowed down hepatic autophagy in vivo. Collectively, our in vitro and in vivo findings suggest that ADH-catalysis of ethanol oxidation produces acetaldehyde that influences the microtubule network and disturbs AV trafficking to lysosomes, thereby disrupting hepatic autophagy.

Acute or binge ethanol exposure induces (accelerates) autophagy (Ding et al., 2010; Thomes et al., 2015), as ethanol-induced oxidant stress suppresses mechanistic target of rapamycin (mTOR) (Ding et al., 2010; Thomes et al., 2013), a major negative regulator of autophagy (Donohue and Thomes, 2014). Interestingly, more robust non-chronic ethanol exposure, such as the one described by Wu et al. (Wu et al., 2012) (2 dose daily for 4 days), inhibits autophagy. This indicates that a condition (ethanol regimen) generating overwhelming levels of oxidant stress within 4 days can block the hepatic autophagy machinery to slow down macromolecular catabolism. Similarly, chronic ethanol creates a continuous condition of oxidant stress to inhibit autophagy, which depends on the levels of oxidants produced during the ethanol exposure regimen (Thomes et al., 2015). Much of the early liver pathology (e.g., steatosis) associated with alcohol abuse can be alleviated by cessation of drinking which could eventually restore autophagy to normal. However, we propose that autophagy is diminished in problem drinkers, thereby contributing to the hallmark features of alcoholic liver disease. Since ethanol exposure disrupts hepatic autophagy, acceleration of autophagy with rapamycin and carbamezipine alleviates chronic ethanol-induced fatty liver and injury in a mouse model of chronic ethanol (Lin et al., 2013). Moreover, a reduction in autophagy has been linked to a variety of liver diseases (Czaja et al., 2013). Thus, there is general agreement that autophagy is a cytoprotective pathway in the liver (Moreau et al., 2010; Czaja et al., 2013). However, it was reported that activation of autophagy in hepatic stellate cells (HSCs) increases fibrogenesis (Hernandez-Gea et al., 2012), and conversely, its inhibition reduces liver fibrosis induced by CCl_4 (Hernandez-Gea et al., 2012). These findings have led some to suggest that blocking HSC autophagy is a viable therapy for liver fibrosis. We demonstrated that alcohol exposure disrupts hepatocyte autophagy (Thomes et al., 2015). Compared with untreated HSCs, 50 mM ethanol or 100 μM Ach exposure for 24 h elevated LC3-II levels in primary rat HSCs (unpublished data). Rat HSCs express ADH1, which suggests that HSCs oxidize ethanol to acetaldehyde leading to higher LC3-II levels. Currently, we are investigating whether ethanol and/or Ach-induced LC3-II represents autophagy acceleration and whether they promote the HSC fibrogenic phenotype by modulating autophagy activity in HSCs. However, we found that exposure of HSCs to the autophagy activator rapamycin or the autophagy inhibitor wortmannin both decreased α-SMA production and cell proliferation in primary rat HSCs (unpublished data). Interestingly, TGF-β-induced fibrogenic phenotype was associated with enhanced cell proliferation but decreased autophagy flux (unpublished data). These latter findings suggest that autophagy per se may not contribute to fibrogenesis. Currently, we are investigating how autophagy activators and autophagy inhibitors both attenuate liver fibrosis. Modulating autophagy is a sensible maneuver to treat liver diseases (Jiang and Mizushima, 2014; Czaja et al., 2013). However, a clear understanding of the functional role of autophagy in advanced liver diseases like fibrosis is essential for targeting autophagy for treatment of liver diseases. Thus, in addition to comprehensive animal model studies, carefully conducted studies are warranted, using surgically- or biopsy-derived liver tissue from human donors to determine the status of autophagy in alcoholic patients. These analyses could lead to practical strategies that use autophagy-modulating agents for the treatment of alcoholic liver disease.

7. Creatinine supplementation: does it prevent alcohol-induced liver injury?

Kusum K. Kharbanda Ph.D.

Previous studies from our laboratory have shown that it is the alcohol-induced reduction in the hepatocellular S-adenosylmethionine (SAM):S-adenosylhomocysteine (SAH) ratio (a.k.a. methylation potential) that impairs the activities of many SAM-dependent methyltransferases (Kharbanda, 2009, 2013). This leads to steatosis and proteasome inhibition (Ganesan et al., 2015; Kharbanda et al., 2007; Kharbanda et al., 2013, Kharbanda et al. 2014; Osna et al., 2010). Guanidinoacetate methyltransferase (GAMT) catalyzes the final reaction in the creatine biosynthetic process. As liver is a major site for creatine synthesis (da Silva et al., 2009) and since GAMT-mediated catalysis consumes as much as 40% of all the SAM-derived methyl groups, creatine production places a substantial methylation burden on the liver (Mudd et al., 2007). We hypothesized that providing creatine exogenously could potentially spare SAM, preserve hepatocellular SAM:SAH ratio and thereby prevent the loss of methylation potential and thus, the development of alcoholic steatosis. Male Wistar rats were pair-fed the Lieber DeCarli control or ethanol diet (Lieber and DeCarli, 1989) with or without 1% creatine supplementation for 4–5 weeks of feeding (Murali et al., 2016). The blood, heart and livers were removed and processed for determining histological and biochemical end-points (Kharbanda et al., 2014). Creatine supplementation neither prevented alcoholic steatosis nor attenuated the alcohol-induced proteasome activity. The hepatocellular SAM:SAH ratio seen in the ethanol-fed rats was also not normalized, when these rats were fed the creatine supplemented ethanol diet. However, a >10-fold increased level of creatine was observed in the liver, serum and hearts of rats fed the creatine-diets. Dietary creatine supplementation did not prevent alcoholic liver injury (Murali et al., 2016) despite preventing choline-deficient or high-fat diet-induced hepatic steatosis (Deminice et al., 2011, 2015). Betaine, that maintains cellular SAM:SAH remains our best option for treating alcoholic steatosis (Kharbanda, 2009, 2013; Thomes et al., 2015).

8. Acetaldehyde a neglected human carcinogen

Mikko Salaspuro Ph.D.

A single point mutation in aldehyde dehydrogenase (*ALDH*)*2*-gene provides conclusive evidence for a causal relationship between acetaldehyde and upper gastro-intestinal tract cancer (Väkeväinen et al., 2000; Maejima et al., 2015). This mutation results in the deficient activity of the mitochondrial ALDH2. When drinking alcohol, ALDH2-deficients are exposed via saliva to 2–3 times and via gastric juice to 5–6 times higher local acetaldehyde concentrations than individuals with the active ALDH2-enzyme. Parallel to the increased local acetaldehyde exposure, the risk of ALDH2-deficient alcohol drinkers for oral, pharyngeal, esophageal and gastric cancer is many fold compared to alcohol drinking ALDH2-actives (Yokoyama et al., 1998; Tsai et al., 2014; Matsuo et al., 2013). Based on the strong epidemiological and biochemical evidence, the International Agency for Research on Cancer (IARC/WHO) has reclassified acetaldehyde associated with the consumption of alcoholic beverages as a group 1 human carcinogen (IARC, 2012). An equivalent human cancer model that is based on the proven gene-biochemical and -environmental interactions is not available for any other of the 118 group 1 human carcinogens. A key factor in

acetaldehyde associated carcinogenesis is its local accumulation after alcohol drinking and tobacco smoking in the upper digestive tract. Normal saliva does not contain measurable levels of acetaldehyde. However, a dose of alcohol results in mutagenic concentrations of acetaldehyde in the saliva, and the enhanced local acetaldehyde exposure continues for as long as ethanol stays in the human body (Homann et al., 1997). Acetaldehyde accumulates in the upper digestive tract due to the local oxidation of ethanol to acetaldehyde by the normal upper digestive tract microbial flora, parotid glands and mucosal cells. However, unlike the liver these organisms and organs are not sufficiently capable for the detoxification of acetaldehyde (Salaspuro, 2003).

Acetaldehyde has a faint apple like aroma. It is soluble to water and lipids and consequently it passes easily the cell membranes. It is carcinogenic to experimental animals. Via its very reactive aldehyde group acetaldehyde has been shown to form mutagenic DNA adducts in the oral mucosa of humans already after a moderate dose of alcohol (Seitz and Stickel, 2010).

Acetaldehyde presumably is the most common human carcinogen. In addition to acetaldehyde formed from ethanol, a high concentration of 'free' acetaldehyde is present in many alcoholic beverages as well as in some foodstuffs produced by fermentation since microbes are able to effectively produce acetaldehyde from ethanol already at very low ethanol concentrations (0.2–1‰) (Balbo et al., 2012; Lachenmeier et al., 2009; Lachenmeier et al., 2010).

Acetaldehyde is widely used as an aroma agent and food additive. It is the most abundant carcinogen of tobacco smoke that dissolves in the saliva during smoking and is by that means distributed to the mucosal surfaces of the whole upper digestive tract (Haussman, 2012; Salaspuro and Salaspuro, 2004). The IARC/WHO has classified acetaldehyde as a group 1 human carcinogen since 2009 (Secretan et al., 2009). The Scientific Committee on Consumer Safety nominated by the European Commission concluded unanimously in 2012 that the maximum concentration for acetaldehyde in cosmetic products is 5 mg/l and that acetaldehyde should not be intentionally used in mouth-washing products (SCCS, 2012). Some alcoholic beverages exceed this concentration over a hundred times and some food over three times. On the contrary, an international scientific expert committee administered jointly by the Food and Agriculture Organization of the United Nations and WHO still considers acetaldehyde to be a Generally Regarded as Safe product. Accordingly there are no restrictions with regard to the use of acetaldehyde as an aroma agent and food additive (JECFA.1998). By limiting alcohol consumption and quitting from tobacco smoking, avoiding beverages and food containing even low levels of ethanol, and maintaining a good oral hygiene people can decrease microbial acetaldehyde production from ethanol by 50–100% (Homann et al., 2001).

Atrophic gastritis is the major risk factor for gastric cancer. It is characterized by a hypochlorhydric or achlorhydric stomach, which is colonized by oral microbes (Salaspuro, 2011). These microbes produce effectively acetaldehyde from any ethanol present in the saliva or gastric juice after consumption of alcoholic beverages or food. Special slowly L-cysteine releasing capsules and lozenges eliminate from 60 to 90% of carcinogenic acetaldehyde from saliva and gastric juice after alcohol administration and tobacco smoking (Salaspuro et al., 2006; Linderborg et al., 2011). These formulations provide a novel approach for the minimization of local acetaldehyde exposure in the upper digestive tract.

9. Alcohol and oral health

Andreea Voinea-Griffin DDS, Ph.D. and Andrei Barasch DMD, MDSc.

Despite a wealth of evidence on the negative impact of alcoholism on digestive tract health, little is known on the link between alcohol abuse and oral health. Numerous studies showed that the relationship between general and oral health is stronger than once believed and sometimes bi-directional (Nagpal et al., 2015). For example, oral morbidities have been associated with low birth weight (Soroye et al., 2015), cardiovascular disease (Abou-Raya et al., 2002), and lung cancer risk (Zeng et al., 2016). Diabetes mellitus and periodontal disease have a bi-directional relationship (Kapellas et al., 2016; Mammen et al., 2016).

Drug-induced salivary and mucosal diseases were long described in the dental literature. Biphosponate-induced osteonecrosis of the jaw has been well documented and changed dental treatment recommendations for those undergoing these treatments (Barasch et al., 2011; Barasch et al., 2003; Vena et al., 2013). de Boissieu and his colleagues (2016) documented bisphosphonate-related osteonecrosis of the jaw in the French national pharmacovigilance database MedDRA among all data from 1985 to 2014 outcome, seriousness in 640 individuals (70% women). Known associated factors for bisphosphonate-related osteonecrosis of the jaw such as dento-alveolar surgery, glucocorticoids, chemotherapy, anti-angiogenics, denosumab, alcohol were identified for 70% of the patients.

Substance abuse has a devastating effect on dental tissues (Shekarchizadeh et al., 2013). Given the relationship between general and oral health, it is possible that alcohol abuse has a larger impact on the health of oral cavity than shown to date.

Caries and periodontitis are the most prevalent oral diseases and share several etiologic factors. Of those, poor oral hygiene, poor diet, decreased salivary flow, and decreased immune response are commonly found in alcoholic patients. Several studies reported on the increased prevalence of periodontal disease (Tezal et al., 2001), coronal caries (Friedlander et al., 2003) and root caries (Hayes et al., 2016) in alcoholic patients. These morbidities are most likely caused by the poor plaque control commonly found when personal hygiene is neglected and the salivary flow is diminished. Neglect and dry mouth are common in patients with high level of alcohol consumption. Research also emphasized the role of age in increasing oral health risk in alcoholic patients (Friedlander and Norman, 2006).

Alcohol abuse has also been associated with erosive tooth wear on the palatal surfaces of the upper anterior teeth (Teixeira et al., 2016). The prevalence of tooth erosions was reported to be as high as 50% and directly associated with the duration of chronic alcoholism. Oral soft tissue lesions are common in alcohol abuse patients, most likely due to the nutritional deficiencies characteristic in this population group. Mucosal ulcers, glossitis, and angular cheilitis are just a few of the orofacial presentations found in patients who abuse alcohol.

Among all oral morbidities, oral cancer has been most clearly associated to alcoholism. Alcohol abuse has been linked to 37% and 17% of oral and pharyngeal cancers in UK men and women, respectively (Parkin, 2012). Alcohol use after an oral cancer diagnosis increases the risk of a second primary tumor by up to 50% (Miller et al., 2006). Concurrent alcoholism and tobacco use results in an increased risk for oral cancer by a factor as high as 35 (Parkin, 2012).

This is a call for research, education, and care coordination with the goal of improving care for the alcoholic patients. Little is known on the mechanisms of the association between alcohol and oral morbidities or whether a causal relationship actually exists. Health care professionals should engage in additional research, be aware of the current knowledge and its limitations, and participate in multidisciplinary teams to better care for alcoholic patients. Dental professionals should be included in these teams, since oral health in alcoholic patients is commonly overlooked. Dental professionals can screen and refer patients for substance abuse interventions, promote oral health, provide preventive dental care, and improve alcoholic patients' ability for food intake. Medical professionals in turn must be suspicious of any oral mucosal lesions and refer for oral cancer screening as soon as a lesion is detected. Knowing the critical role of nutrition and the emergency care seeking pattern in this patient population, referral for dental care is an important step in maintaining not only oral but also general health. Health care professionals should integrate care across medical and dental disciplines if better outcomes are to be achieved. Moreover, the relationship between alcohol abuse and oral health is strong and may warrant a concerted effort of the research community.

10. Alcohol and colorectal cancer

Helmut K. Seitz M.D.

The International Agency for Research on Cancer declared alcohol as a risk factor for colorectal cancer (Baan et al., 2007a, 2007b), since epidemiological case control- as well as prospective cohort and correlation studies have demonstrated significant correlation between alcohol intake and colorectal cancer risk with a dose-response relationship (Seitz and Homann, 2012). In most of the animal experiments in which a carcinogen was given to induce colorectal cancer the additional administration of alcohol increased tumor yield. Furthermore the administration of alcohol as 20% in drinking water for ten weeks increased intestinal tumors in the C57/B6 ABC-min mouse (Roy et al., 2002). In addition, when a local carcinogen which does not need metabolic activation was applied to the colon and colorectal mucosa in animals, an acceleration of carcinogenesis was observed (Seitz et al., 1990). Alcohol is delivered from the blood to the colon and reaches the same levels in the colon content as in blood. Alcohol is metabolized in the colon mucosa by alcohol dehydrogenase (ADH) and by bacterial enzymes in both cases to acetaldehyde. The highest levels of acetaldehyde occur in the rectum since bacteria have a high capacity to oxidize alcohol to acetaldehyde (Seitz et al., 1990). Acetaldehyde concentrations in the colorectum correlate significantly with cell cycle behavior. Acetaldehyde leads to a hyperproliferation of the mucosa and to an extension of the proliferative compartment of the crypt towards the lumen which resembles an early risk for cancer. Similar observations as in rats have also been observed in men (Simanowski et al., 2001). Since the ADH1C1 allele codes for an enzyme which has a 2.5 times faster metabolic rate to produce acetaldehyde, it was not surprising that an alcoholic patient who consumes >30 g alcohol per day with the genotype ADH1C1,2 has a significantly increased risk for colorectal cancer (Homann et al., 2009). In addition, it was observed that crypt-cell behavior was also affected by vitamin E (Vincon et al., 2003). Since vitamin E inhibits alcohol mediated hyperproliferation it is suggested that oxidative stress may play a role. In the alcoholic most of the oxidative stress comes from the induction of cytochrome P450 2E1 (CYP2E1) which metabolizes ethanol. As a side reaction reactive oxygen species (ROS) occur which may lead to lipid peroxidation and finally generation of highly carcinogenic exocyclic etheno-DNA adducts (Linhart et al., 2014). This has been shown in the liver and the upper gastrointestinal tract.

In a recent study with 42 alcoholics and 12 control patients we determined CYP2E1 as well as etheno DNA-adducts in the colon mucosa by immunochemistry. There was a great variability in the presence of both CYP2E1 and etheno DNA adducts. However, no significant difference between alcohol and control patients was found, while a significant correlation between CYP2E1 and εdA was observed. Since alcohol consumption leads to apoptosis at least in isolated intestinal cells (Wu and Cederbaum, 2004) we wonder whether this is also the case in humans. Therefore, we determine apoptosis and anti-apoptotic protein in our patients. While inflammation and apoptosis was absent in all biopsies, the anti-apoptotic protein Mcl-1 was found to be significantly increased. Mcl-1 has a short half life and has been identified as the key-protein responsible for rapidly changing environmental cue conditions. Mcl-1 has functions beyond cell dysregulation; a regular contribution of Mcl-1 to invasiveness, cell cycle and mitochondrial respiration has been described (Koehler et al., 2015). The survival benefits in colorectal mucosa gained through up-regulated Mcl-1 might end up in a cell with accumulated DNA-damage and mutation and may facilitate carcinogenesis.

11. Biomarkers in nonalcoholic fatty liver disease

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Nonalcoholic fatty liver disease (NAFLD) refers to the lipido-hepatocyto-toxicity when no other causes for fat accumulation in hepatocytes is declared or known such as heavy alcohol consumption (Zimmerman, 1999), drug-induced (Neuman et al., 2015a) or herbal-induced liver injury (Neuman et al., 2015b, 2015d). NAFLD is increasingly prevalent affecting children, adolescents and adults, leading to development of atherosclerosis and the metabolic syndrome (MS), both of which significantly increase the risk of cardiovascular disease (CVD) and non-alcoholic steatohepatitis (NASH) with morbidity and mortality (Bellentani and Marino, 2009; Argo and Caldwell, 2009; Neuman et al., 2014a). NAFLD is characterized by insulin resistance frequently associated with hepatic fat accumulation. In NAFL, hepatic steatosis is present without evidence of inflammation, whereas in NASH, hepatic steatosis is leading to severe steatohepatitis with centrilobular necro-inflammation. This inflammation histologically is indistinguishable from alcoholic steatohepatitis. NASH inflammation shows hepatocyte injury (ballooning) and Mallory-Denk bodies with or without fibrosis. NASH is most common in middle-aged persons but is found in all age groups. NASH typically occurs in persons who are overweight (Neuschwander-Tetri, 2010) or diabetic (Fagot-Campagna et al., 2000), but it has recently

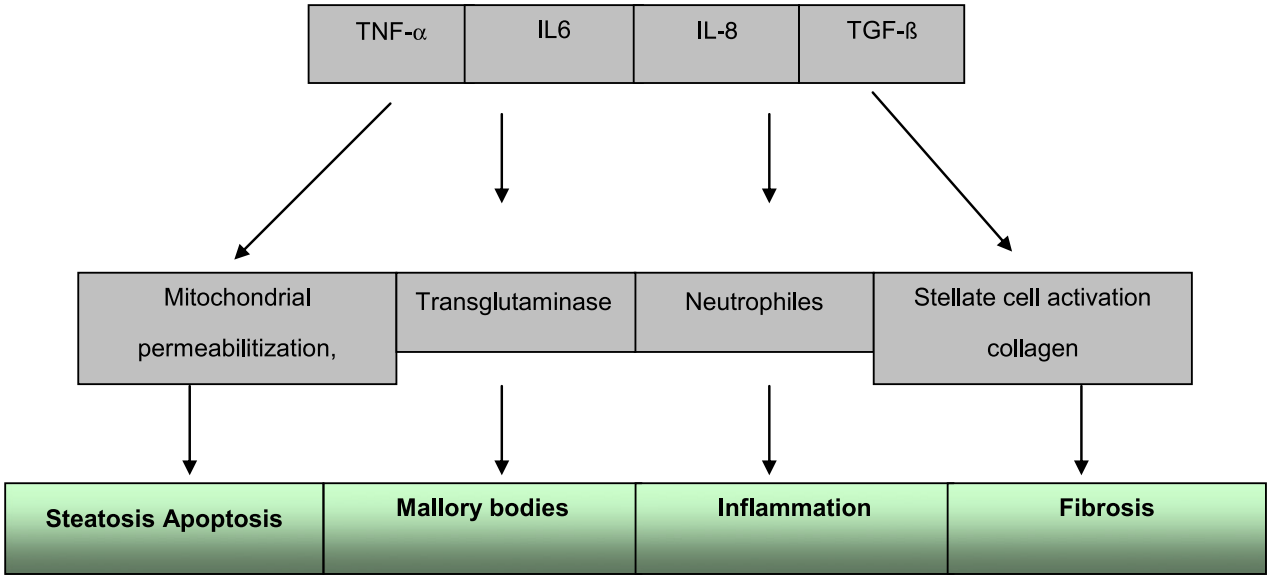

Fig. 7. Lipid peroxidation — reactive oxygen species

been shown to occur in subjects with normal body weight and normal glucose tolerance (Neuman et al., 2014b). Familial tendency to NASH-induced hepatocellular carcinoma (HCC) has been described by our group (Neuman et al., 2005). The increased prevalence of cirrhosis and HCC in diabetes and obesity has lead to consider NAFLD as the main cause of a raising incidence of liver complication and liver related death in patients with these clinical conditions.

Both excessive BMI and visceral obesity are recognized risk factors for NAFLD. In patients with severe obesity undergoing bariatric surgery, the prevalence of NAFLD can exceed 90% and up to 5% of patients may have unsuspected cirrhosis (Boza et al., 2005; Haentjens et al., 2009; Machado et al., 2006; Colicchio et al., 2005; Beymer et al., 2003). There is a very high prevalence of NAFLD in individuals with type 2 diabetes mellitus (T2DM) (Marchesini et al., 2001; Vernon et al., 2011). Moreover, Weikert and Pfeiffer (2006) show glucose metabolism in the liver signals for fatty infiltration in the liver. Troiano and Flegel (1998) make the link between obesity and diabetes in children. An ultrasonographic study of patients with T2DM showed a 69% prevalence of NAFLD (Leite et al., 2009). In another study, 127 of 204 diabetic patients displayed fatty infiltration on ultrasound, and 87% of the patients with histological confirmation of NAFLD (Prashanth et al., 2009).

Elevated serum alanine amino transferase (ALT) concentration has been used to estimate the prevalence of liver disease using pooled data from the National Health and Nutrition Examination Survey (NHANES) 1999–2004, which included 14,855 adult participants. Using the definition of abnormal ALT (>30 IU/ml for men and >19 IU/ml for women) (Prati et al., 2002), 41.7% of adult NHANES participants were found to have liver disease. The adult NHANES III data set demonstrated that 69% of all ALT elevations were not explained by viral hepatitis, alcohol consumption, or hereditary hemochromatosis (Clark et al., 2003). The authors concluded that NAFLD is probably responsible for the majority of cases of liver disease.

However, Suzuki et al. (2005) show chronological development of elevated aminotransferases in a nonalcoholic population. Other terms that have been used to describe NASH include pseudoalcoholic hepatitis, alcohol-like hepatitis, fatty liver hepatitis, steatonecrosis, and diabetic hepatitis (Zimmerman, 1999). NAFLD is subdivided into nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). In NAFL, liver fatty inclusions are present without inflammation, whereas in NASH, fatty inclusions in hepatocytes are associated with inflammation. The picture is morphologically indistinguishable from alcoholic steatohepatitis (ASH). NAFLD does not require a liver biopsy. However, liver biopsy is the only confirmation or exclusion of NASH and the only way to determine disease severity. The NAFLD activity score (NAS) is the sum of the biopsy's individual scores for steatosis (0 to 3), lobular inflammation (0 to 2), hepatocellular ballooning (0 to 2), and fibrosis (0 to 4). An NAS <3 corresponds to NAFL, 3 to 4 corresponds to borderline NASH, and a score ≥5 corresponds to NASH (Kleiner et al., 2005; Brunt and Tiniakos, 2010; Brunt et al., 1999). NASH is commonly associated with perisinusoidal and perivenular fibrosis that may progress to cirrhosis. About 30–40% of patients with NAFLD develop NASH. Moreover, it is estimated that 10–30% of patients with NAFLD develop cirrhosis after 10 years, leading to hepatocellular carcinoma (NASH is believed to be a mitochondrial disease arising from the inability of the mitochondria to adapt to fat oversupply (Caldwell et al., 2004). The following schematic representation presents how lipid peroxidation generated by reactive oxygen species influence the inflammatory status by activating and releasing inflammatory and profibrotic cytokines (Fig. 7).

Therapeutic interventions may reduce hepatic steatosis and the development of necro-inflammation/fibrosis by reversing defects at 3 levels: 1) reducing substrate supply for lipogenesis a) from excess dietary triglycerides, or b) from excessive lipolysis and free fatty acid [FFA] flux to the liver from insulin-resistant adipose tissue; 2) activating key molecular steps that stimulate fatty acid oxidation and/or inhibit hepatic lipogenesis (i.e., AMP-activated protein kinase [AMPK]); or 3) by ameliorating the inflammation cascade generated by mitochondrial dysfunction from fat overload (i.e., activation of Kupffer cells, local production of cytokines, etc.).

The greater risk of progression of liver disease and the additional cardiovascular risks associated with NASH provide the rationale for identifying patients who have NASH. These considerations have led to intense interest in the development of noninvasive methods for the diagnosis, grading, staging and follow up of patients with NASH. While several panels have been developed, they lack the diagnostic accuracy required for wide scale application. Therefore there is an immediate need for a noninvasive method for evaluating and monitoring the progress of NASH. Identifying the severity of liver function in patients with NAFLD including those with NASH is a major problem in decision-making in clinical hepatology.

The majority of the NAFLD/NASH patients in North America do not drink actively, but they misuse alcohol sometime in their lifetime and a possible alcohol-induced liver damage was triggered in that period of time. Moreover, some do not consider dangerous drinking if they have one of two episodes of alcohol misuse. The quantitative, measurable detection of drinking is important for the successful diagnosis and treatment of alcohol misuse as well as NAFLD/NASH many of whom continually deny drinking. The accurate identification of alcohol consumption via biochemical tests contributes significantly to the monitoring of drinking behavior both in ASH and NAFLD/NASH.

Rinella and Sanyal (2016) estimated the prevalence of NAFLD in the USA to be 30% of the population. Therefore there is a real need for a reliable, non-invasive method to distinguish between NAFLD and NASH in order to identify those patients most at risk of adverse outcomes and to

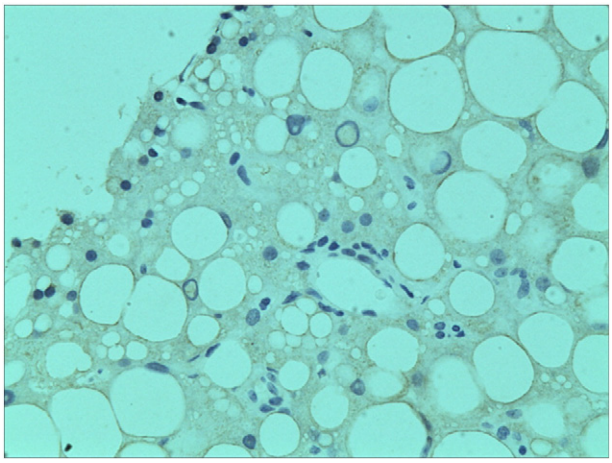

Fig. 8. Immunohistochemical staining of a precursor caspase is clearly represented in a biopsy of a patient with NASH. Hepatocytes presenting macro-vesicular steatosis can be seen. Very few hepatocytes present microvesicular steatosis. Apoptotic bodies can be observed (black spots).

Biomarkers for alcohol ingestion have been recently reviewed by our group (Nanau and Neuman, 2015). Alcohol ingestion can be measured using a breath test. Because alcohol is rapidly eliminated from the circulation, the time for detection by this analysis is in the range of hours. Alcohol consumption can alternatively be detected by direct measurement of ethanol concentration in blood or urine. Several markers have been proposed to extend the interval and sensitivities of detection, including ethyl glucuronide and ethyl sulfate in urine, phosphatidylethanol in blood, and ethyl glucuronide and fatty acid ethyl esters in hair, among others. Moreover, there is a need to correlate the indirect biomarker carbohydrate deficient transferrin, which reflects longer lasting consumption of higher amounts of alcohol, with serum γ-glutamyl transpeptidase, another long term indirect biomarker that is routinely used and standardized in laboratory medicine. Therapeutic interventions may reduce steatosis and the development of inflammation by reducing substrate supply for lipogenesis from triglycerides, or from excessive lipolysis and free fatty acid (FFA) flux to the liver from adipose tissue. In addition the target of therapeutic intervention can inactivate the inflammation cascade generated by mitochondrial dysfunction, activation of Kupffer cells, or increase release of pro-inflammatory cytokines.

provide them with the relevant information needed in order to make the required lifestyle adjustments. More importantly there is a need to recognize the severity of the disease using non-invasive biomarkers, with particular emphasis on personalized pharmacologic therapy.

In 1998, the National Institutes of Health (NIH) Biomarkers Definitions Working Group BDWG defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention."

A joint venture on chemical safety, the International Program on Chemical Safety, led by the World Health Organization (WHO) and in coordination with the United Nations and the International Labor Organization, has defined a biomarker as "any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease."

The definition of biomarkers includes "almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological. The measured response may be functional and physiological, biochemical at the cellular level or a molecular interaction. Understanding the clinical importance of biomarkers that indicate the severity of NAFL and the more severe stage NASH as well as following the biomarkers' status in time (kinetics) is a possible therapeutic endeavor. In clinical safety assessment, compounds in early development of therapeutics often show signs of toxicity during clinical trials. The use of biomarkers, and in particular laboratory-measured biomarkers, in clinical research is somewhat newer, and the best approaches to this practice are still being developed and refined. The key issue at hand is determining the relationship between any given measurable biomarker and relevant clinical endpoints. An essential element of biomarkers used for clinical decision-making is that the marker is clinically relevant and clinically valid. The challenge has to identify the mechanism of the disease progression from NAFLD to NASH, as well as, biomarkers, which can be developed into targeted assays. In our studies we used as a biomarker of apoptosis in NASH-cleaved caspase cytokeratine 8 (CCK18-M30) correlating the levels in sera and in the biopsy of the same patient.

Immunohistochemical staining of a precursor caspase is clearly represented in a biopsy of a patient with NASH (Fig. 7). The field of biopsy contains hepatocytes presenting macro-vesicular steatosis. Very few hepatocytes present microvesicular steatosis.

Also NAFL/NASH individuals have a cytokine profile that is different from healthy individuals and from one condition to the other. There is a correlation between cytokines and the severity of the disease. Moreover, the cytokine profile is altered during the course of therapy. Cytokine levels in sera can be used to predict the severity of the disease, to monitor the progression of the disease and to predict the outcome of the therapy (Fig. 8).

Previously, assessment of the MS and NAFLD has involved the analysis of serum or plasma biomarkers including total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), insulin, and C-peptide.

Miele et al. (2009) considered serum levels of hyaluronic acid and tissue metalloproteinase inhibitor-1 combined with age to predict the presence of nonalcoholic steatohepatitis in a pilot cohort of subjects with nonalcoholic fatty liver disease. More recently, biomarkers such as apolipoprotein (apo)-AI and apo-B have been proposed as predictors.

Similarly, leptin, adiponectin, free fatty acids (FFA), and ghrelin are emerging biomarkers of insulin resistance (Friedman and Halaas, 1998; Halaas et al., 1995; Silha et al., 2003; Trujillo and Scherer, 2005). Of the latter group, adiponectin, ghrelin, and free fatty acid (FFA) have also been implicated as biomarkers of insulin resistance and NAFL (de Jongh et al., 2004; Ouchi et al., 1999; Katugampola et al., 2002).

Adipokines derived from visceral adipose tissue are delivered directly to the liver via the portal vein (Eguchi et al., 2006). Adiponectin is an anti-inflammatory cytokine. Hypo-adiponectinemia has been suggested to play a role in the progression from NAFLD to NASH (Musso et al., 2005). Also, our studies have indicated associations between inflammation in NASH and serum levels of inflammatory cytokines including tumor necrosis factor-α (TNF-α) and fibrosis with transforming growth factor beta (TGF-b). Relevance on the contribution of adipokines in inflammation and repair of liver damage produced by lipids continues to be our translational research aim. We describe recently adipokine levels in patients with biopsy-proven NAFLD and NASH, showing that these adipokines are associated with liver histology and more specifically with the degree of liver steatosis (Neuman et al., 2015a).

An additional marker, circulating resistin levels were positively associated with histological steatosis, portal inflammation and NAS in patients with NAFLD and NASH (Pagano et al., 2006). The study groups of Senates et al., 2012 and Milner et al. (2009) suggested that adipocyte-fatty acid binding protein (AFABP) may play a role in NAFLD progression. The authors indicated that serum AFABP is positively correlated with inflammation, ballooning and fibrosis in non-obese patients with NAFLD. Also AFABP had a positive association with lobular inflammation, hepatocellular ballooning and NAS. Hepatocellular ballooning remained independently associated with AFABP on multiple linear regression also correcting for age, BMI, fasting glucose, total cholesterol, triglyceride, steatosis and fibrosis (Shen et al., 2012).

Another adipokine, vaspin was suggested to associate with liver histology in studies with biopsy-proven NAFLD patients. Kukla et al. (2010) found a positive correlation between serum vaspin levels and cell ballooning in obese NAFLD individuals. Also, Aktas et al. (2011) reported that vaspin is correlated with liver fibrosis. However, vaspin levels were not correlated with histology in non-diabetic non-obese NASH individuals (Genc et al., 2011, 2013).

Biomarkers such as apelin-12 (Ercin et al., 2010) and apelin-36 (Aktas et al., 2011), could not be correlated with histology.

Bozaoglu et al. (2007) described chemerin to be associated with obesity and metabolic syndrome, while Takahashi et al. (2008) observed that chemerin enhances insulin signaling and potentiates insulin-stimulated glucose uptake in cultured adipocytes. Moreover, Krautbauer et al. (2013) observed that chemerin is highly expressed in hepatocytes and is induced in NASH-liver. Yilmaz et al. (2011) proposed serum levels of omentin, chemerin and adipsin to be measured in patients with biopsy-proven NAFLD. Monitoring disease progression or repair by following changes in cyto-adipokine levels is a new strategy that does not exclude liver biopsy, but together with imaging and clinical examination can reduce the frequency of histological examination.

Moreover there are functional tests that have been proposed to be used in NAFLD/NASH such as: (13)C-Octanoate Breath Test (Miele et al., 2003).

While several therapeutic strategies have been proposed to improve this condition, there are also non-medicinal interventions used to reduce liver involvement or to prevent the disease altogether. The likely development of effective therapies e.g. thiazolidinediones for NASH provides further impetus for the identification of those with risk to disease progression. Although pharmacological therapy has been tried it is only partially successful and the cornerstone of successful therapy consists of weight loss and physical exercise. Thus therapy of this common condition requires for most patients a decision to change their lifestyle (Neuman et al., 2015c). Lifestyle intervention is important for all patients irrespective of NAFL/NASH stage. This is not easy to achieve and maintain in the long term. Although much progress has been made in the past decade with respect to understanding NAFL/NASH and developing partially effective therapies, much more needs to be learned about disease pathogenesis as this is the key to developing more broadly effective management strategies and treatments. These therapies should be targeted to the individuals knowing the specific stage of the disease those most likely to benefit from the specific intervention knowing the risk factors for disease progression.

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